Journal of Medical Virology 58:353–358 (1999)

Mutations in the NS5A Region Do not Predict Interferon-Responsiveness in American Patients Infected With Genotype 1b Hepatitis C Virus

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A striking association has been demonstrated recently between mutations in amino acid residues 2209–2248 of the nonstructural protein 5a (NS5a) region of hepatitis C virus (HCV) and sustained responses to interferon in Japanese patients infected with genotype 1b. Therefore, analysis of this sequence has been suggested as a predictor of treatment response. We sought to determine whether mutations in this region predict outcome in U.S. patients infected with genotype 1b hepatitis C virus (HCV-1b). We analyzed stored pretreatment sera retrospectively from 22 patients with HCV-1b infection who had received interferon alpha-2b (IFNα-2b) as part of a controlled trial. Two patients were sustained responders (SR), 7 were transient responders (TR), and 13 were nonresponders (NR). We performed nested reverse transcription-polymerase chain reaction (RT-PCR) on extracted RNA using primers flanking HCV amino acids 2209–2248 and sequenced the PCR products directly. The deduced amino acid sequences were compared with the prototype HCV-J. Isolates with four or more deviations from the prototype were defined as "mutant" type, those with one to three substitutions as "intermediate" type, and those matching the prototype as "wildtype." Of the 22 HCV-1b isolates, 6 were wildtype, 11 intermediate type, and 5 mutant type. Both of the SRs were intermediate type. The 20 TRs and NRs were distributed among mutant (5), intermediate (9), and wildtype (6). Of the five patients with mutant virus, four were NR and one a TR. Variation in $NS5a_{2209–2248}$ fails to predict interferon responsiveness in this cohort of American patients infected with HCV-1b. Thus, the utility of this sequence as a predictor of interferon responsiveness appears to be specific to Japanese patients and may reflect differences between patient groups in treatment regimens, host genetic background, or alterations in the interferon signaling pathway induced by surrounding sequences within or outside NS5a. Overall, NS5a is not as integral a determinant of interferon responsiveness as previously suggested. ***J. Med. Virol. 58:353–358, 1999.***

KEY WORDS: hepatitis C virus; NS5a; interferon sensitivity determining region; genotype 1b

INTRODUCTION

Hepatitis C virus (HCV) infection is the most common cause of parenterally transmitted hepatitis worldwide. Infection with this virus leads to chronic liver disease in more than 80% of cases, and progression to cirrhosis and hepatocellular carcinoma occurs in a significant number of infected individuals. As a result, chronic hepatitis C is the leading indication for liver transplantation in the United States [Detre et al., 1996].

Interferon-alpha (IFN-α), either alone or in combination with ribavirin, is currently the only approved therapy for chronic HCV infection. The overall effectiveness of this agent, however, has been suboptimal; sustained responses to IFN monotherapy occur in only 10–20% of treated individuals [Hoofnagle and Di Bisceglie, 1997]. Compounding its limited efficacy are the considerable cost, inconvenient delivery, and high frequency of side effects associated with IFN. Pending the development of more effective therapies, investigators have focused attention on defining factors that predict a favorable response to IFN and that might be used to

Presented in part at the meeting of the American Association for the Study of Liver Diseases in Washington, D.C. (May 1997).

Grant sponsor: National Institutes of Health; Grant numbers: K08-DK02209 (to RTC), R01-AI33455 (to LMK); Grant sponsor: Molecular Biology Core of the MGH Center for the Study of Inflammatory Bowel Disease; Grant number: P30-DK43351.

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Accepted 18 December 1998

TABLE I. Genotype-Specific Oligonucleotide Primers Used for PCR of $NS5a_{2209–2248}$

1b upper outer (1bUO)	5′ - T G G A T G G A G T G C G G T T G C A C A G G T A - 3′	(nt 6703–6727)*
1b lower outer (1bLO)	5′ - T C T T T C T C C G T G G A G G T G G T A T T G G - 3′	(nt 7296–7320)
1b upper inner (1bUI)	5′ - C A G G T A C G C T C C G G C G T G C A - 3′	(nt 6722–6741)
1b lower inner (1bLI)	5′ - G G G G C C T T G G T A G G T G G C A A - 3′	(nt 7275–7294)
1a upper outer (1aUO)	5′ - A T C G C C C G A A T T T T T C A C A G - 3′	(nt 6692–6711)
1a lower outer (1aLO)	5′ - C G T A G T C G G G C T T T T T C C A C - 3′	(nt 6900–6919)
1a upper inner (1aUI)	5′ - G C G G G A G G A G G T A T C A T T C A - 3′	(nt 6764–6783)
1a lower inner (1aLI)	5′ - A G A C T T C C G C A G G A T T T C T G - 3′	(nt 7157–7176)

*Sequence based on numbering for HCV-J (Kato et al., 1990).

identify patients most likely to benefit from treatment. Such positive predictive factors identified thus far include infection with HCV genotypes other than genotype 1, low concentrations of circulating viral RNA, brief duration of disease, low quasispecies diversity, and absence of cirrhosis [Kanazawa et al., 1994; Tsubota et al., 1994; Martinot-Pegnoux et al., 1995; Yamada et al., 1995; Le Guen et al., 1997]. Unfortunately, however, in the United States and most of Europe, HCV infection with genotype 1 predominates, and this genotype is associated with poor sustained response rates. Similarly, the predictive value of the other variables is insufficient to determine whether or not to use interferon in a given clinical setting [Davis and Lau, 1997].

Recently, Enomoto et al. [1995, 1996] found a striking correlation between the presence of amino acid (aa) substitutions in a short segment of the HCV nonstructural protein 5a (NS5a) and a sustained response to IFN in persons infected with HCV genotype 1b (HCV-1b) [Enomoto et al., 1995, 1996]. These patients had received 6–10 million units of IFN-α for 6 months (total dose 516–880 MU). The presence of four or more amino acid deviations ("mutations") from the prototype 1b sequence within a 40-aa segment of NS5a (aa 2209–2248) was associated with a sustained response in all 16 patients studied. Conversely, a sustained response was seen in none of the 30 patients whose NS5a sequence matched the prototype strain completely ("wildtype"). The predictive value of this sequence, which the authors termed the "interferon sensitivity-determining region" (ISDR), was independent of other predictive variables, including the level of hepatitis C viremia. Thus, this finding implied the existence of a viral factor (genetic element or protein product) that determines or reflects IFN sensitivity. The existence of two phosphorylation sites in the putative ISDR raised the provocative possibility that this region may include a site important for regulating viral replication or virus–host interactions [Tanji et al., 1995]. Because of the potential biological importance of this region, we sought to determine whether the mutated sequence is broadly predictive of IFN sensitivity. As shown below, we have found that in U.S. patients infected with HCV-1b, this sequence has no bearing on IFN responsiveness.

MATERIALS AND METHODS

Patients

We analyzed stored sera from 27 patients with chronic hepatitis C infection who had received recombinant IFN-α-2b in 1990–1991 at the Massachusetts General Hospital as part of a controlled clinical trial [Lindsay et al., 1996]. Written informed consent was obtained from each patient and the study protocol conformed to the Helsinki guidelines. In each case, the initial diagnosis of HCV was made by first generation enzyme immunoassay (Ortho Diagnostics, Raritan, NJ) and confirmed by first-generation recombinant immunoblot assay. Each patient had biopsy-proven chronic hepatitis, and each received treatment with recombinant IFN-α-2b (Intron A, Schering-Plough, Kenilworth, NJ), at least 3 million units subcutaneously thrice weekly for 24 weeks (total dose 216–720 MU). Pretreatment sera that had been collected on all patients and stored at −80°C without thawing were analyzed.

Patients enrolled in the protocol were monitored monthly with serial alanine aminotransferase (ALT) determinations during treatment and for 6 months following completion of therapy. Those patients whose ALT levels were normal with clearance of HCV RNA at the end of therapy were considered responders. Sustained responders (SR) were defined as those who had consistently normal ALT measurements and negative HCV RNA throughout the completion of the 6-month follow-up period, whereas transient responders (TR) experienced a recurrent rise in serum ALT during the follow-up period. Nonresponders (NR) were defined as patients whose ALT levels or HCV RNA failed to normalize by the completion of therapy.

Genotyping of HCV in Patient Sera

RNA was extracted from serum samples using the Ultraspec RNA method (Biotecx, Houston, TX). Reverse-transcription-polymerase chain reactions (RT-PCR) were performed on these RNA preparations using a nested approach (RNA PCR, Perkin Elmer Cetus, Norwalk, CT). Viral genotyping was performed using RT-PCR with separate sets of genotype 1a- and 1b-specific primers corresponding to the region encoding NS5a [Choo et al., 1989; Kato et al., 1990] followed by direct sequencing of the predicted products. In no case did serum that tested positive by one set of primers also test positive for the other set. Sequencing of the RT-PCR products confirmed the genotype of the viral isolates.

The sequences of the primers used for the nested PCR are shown in Table I. Sera from patients whose analysis revealed infection with HCV-1b ($n = 22$) were selected for further analysis.

TABLE II. $NS5a_{2209-2248}$ Sequences From Genotype 1b Sera

Genotype 1b sequences	Number of nucleotide substitutions	Clinical response*	[HCV], MEq/ml.
Prototype			
PSLKATCTTHHDSPDADLIEANLLWRQEMGGNITRVESEN			
Wildtype			
--	0	NR	2.88
--	0	NR	1.99
--	0	TR	0.30
--	0	NR	0.67
--	0	NR	0.30
--	0	TR	0.052
Intermediate type			
F----------V----------------------------	2	NR	2.96
L----------V----------------------------	2	NR	0.29
F----------V---T------------------------	3	NR	<0.001
F----------V---T------------------------	3	SR	0.78
L----------V---T------------------------	3	TR	0.004
F----------V---T------------------------	3	NR	1.89
-----------V----------------------------	1	TR	0.009
-----------V----------------------------	1	TR	1.22
F-----N--------T------------------------	3	NR	0.25
L-----------A--T------------------------	3	TR	4.92
L-----------A--T------------------------	3	SR	0.90
Mutant type			
AF-EG------V----------------------------	5	NR	0.029
L----------GA--T------------------------	4	NR	0.38
L-----N----GA--T------------------------	5	NR	3.00
L----G-----V---T------------------------	4	NR	1.06
VFVEGK-N--SGA-EN------------------------	12	TR	2.12

*SR, sustained response; TR, transient response; NR, no response.

Analysis of NS5a Sequences From Patient Sera

To determine the nucleotide sequence of the NS5a segment of interest, we amplified the region from nucleotides 6722–7294 (numbering as described in strain HCV-J) using the inner pair of 1b primers. Five percent of the initial amplification reaction with the outer primer pair was amplified further in the reaction with the nested primer pair.

Reverse transcription was performed at 42°C for 15 min followed by boiling for 5 min. Following addition of 1U of Taq polymerase, 30 cycles of PCR were performed under the following thermal cycling conditions: 95°C for 60 sec, 45°C for 60 sec, and 72°C for 60 sec. After confirming the generation of appropriately sized DNAs by agarose gel electrophoresis, we gel-purified the PCR products (QIAquick, Qiagen, Chatsworth, CA) and sequenced them directly using an automated DNA sequencer (Applied Biosystems Prism, Model 377). Both DNA strands were sequenced by initiation with the upper and lower inner PCR primers, respectively. Sequences of the predicted DNA translation products were compared to the prototype NS5a sequence from the HCV-J strain (genotype 1b) [Kato et al., 1990]. Conforming with the classification proposed by Enomoto et al. [1996], isolates with four or more aa deviations from the prototype sequence in this region were defined as the mutant type, those with one to three substitutions as intermediate type, and those identical to the prototype as wildtype.

Quantitation of HCV RNA

Serum HCV RNA levels were assessed using the Amplicor quantitative PCR assay (Roche Molecular Diagnostics, Nutley, NJ). Results were expressed in millions of viral genome copies (MEq) per milliliter. The lower limit of detection was 0.001 MEq/ml.

RESULTS

Of the 27 patients enrolled in the trial, 22 were infected with genotype 1b virus and 5 with genotype 1a virus, as determined by RT-PCR with genotype-specific primers. Sequence analysis of the amplified products confirmed the genotype assignments. The amino acid sequences of the NS5a aa 2209–2248 region from the patients infected with HCV-1b are shown in Table II.

Overall, among our 22 patients infected with genotype 1b virus, 9% were SRs, 32% were TRs, and 59% were NRs.

Table II shows the treatment response of individual patients infected with genotype 1b virus, grouped by NS5a sequence. Of the 22 viral isolates, 6 were wildtype, 11 were intermediate, and 5 were mutant-type. Neither of the 2 SRs possessed the mutant-type sequence; both possessed intermediate-type sequences. Of the 7 TRs, 1 harbored virus with mutant-type sequences, 4 had intermediate, and 2 had wildtype sequences. Isolates from 4 of the 13 NRs were mutant type, 5 were intermediate type, and 4 were wildtype.

Conversely, of the 5 patients infected with mutant-type virus, *none* was an SR; 4 were NRs, and 1 was a TR.

When HCV RNA levels were analyzed separately (Table II), we found a trend but no statistically significant correlation between the likelihood of achieving a sustained response and the level of viremia. Both SRs had circulating HCV RNA levels of <1 MEq/ml but did not differ appreciably in level of viremia from the remainder of the group. We found no correlation between HCV RNA levels and the number of substitutions in the NS5a aa 2209–2248 sequence. Given the very small number of sustained responses in this group, we were not in a position to test the relation between viral load and IFN responsiveness.

DISCUSSION

Potentially, the observation by Enomoto et al. [1996] of a nearly complete correlation between the presence of amino acid substitutions within a short segment of the HCV NS5a protein and IFN sensitivity should have had profound implications for the management of patients with hepatitis C. The existence of a virus-specified IFN response determinant, if present, would also shed light on the mechanism of IFN-induced suppression of HCV replication. Because the reported observations were limited to Japanese patients infected with HCV-1b, we sought to assess the generalizability of this observation in other populations of HCV-infected individuals.

Our retrospective analysis of U.S. patients infected with HCV-1b treated with IFN-α demonstrated that deviations in the sequence of NS5a between amino acids 2209 and 2248 did *not* predict responsiveness to IFN. Specifically, none of the five patients infected with HCV whose NS5a sequence deviated from the prototype sequence by four or more amino acids experienced a sustained response to this antiviral agent. Interestingly, the two SRs in this group were infected with HCV whose sequence in this region varied from the prototype by three amino acids, nearly meeting the criterion for mutant-type virus. However, our patients infected with intermediate-type strains were distributed evenly among SRs, TRs, and NRs, underscoring the failure of this NS5a sequence to predict IFN responsiveness in the population studied.

Our results differ from those of Enomoto and other Japanese investigators [Enomoto et al., 1996; Chayama et al., 1997; Kurosaki et al., 1997], who showed that multiple substitutions in this NS5a gene segment of HCV-1b correlated nearly completely with sustained responsiveness to IFN. Rather, our findings are similar to those in recent reports from European groups that failed to demonstrate such a correlation [Squadrito et al., 1997; Zeuzem et al., 1997]. In fact, in one European study [Zeuzem et al., 1997], the post-treatment isolates of three patients who failed to respond to IFN revealed the presence of more mutations in the NS5a region than in pretreatment sequences. This evolution toward a more "mutant" sequence in nonresponders bolsters the evidence against the correlation between the presence of this sequence and treatment response. Taken together, the data suggest that the utility of the $NS5a_{2209–2248}$ sequence as an independent predictor of IFN treatment outcome is limited to Japanese patients and not generalizable to other populations.

Although the sample size is not large and therefore the possibility of a type II statistical error cannot be excluded, our study is the largest reported to date of American patients infected with HCV-1b. In another, smaller study, six patients with genotype 1b infection were described, one of whom was a sustained responder [Hofgartner et al., 1997]. This patient was infected with a strain with two amino acid substitutions compared to prototype; two transient responders were also described with three and one substitution, respectively. However, none of these six subjects had the mutant type strain of HCV-1b. The same group more recently described an additional two patients with genotype 1b infection, one of whom was a nonresponder but who possessed a major variant species with 5 amino acid substitutions; the other was a transient responder with two substitutions [Polyak et al., 1998]. Our findings therefore confirm the suggestion of this North American study that there is no correlation between substitutions in the $NS5a_{2209–2248}$ region and IFN responsiveness in genotype 1b strains in the American population. Overall, then, North American genotype 1b infection appears to exhibit similar clinical behavior as European 1b infection.

The basis for the striking difference between the studies of Japanese and Western patients is not clear. Among the possibilities are subtle differences in viral strains that are not reflected in the sequences used for genotyping, differences in treatment regimens, and host factors such as genetic background or predisposition to chronicity. Although higher cumulative doses of IFN-α have typically been given in Japan, the development of sustained responses in two patients with three amino acid substitutions (intermediate type) and not in any of our patients with mutant-type sequences is difficult to explain on the basis of dose considerations alone. Another consideration is the possibility that viral sequences determined in studies of NS5a mutations may not reflect the diversity of quasispecies at the time of treatment. Some investigators have suggested that clinical outcomes may be better predicted by assessment of strain diversity than by primary sequence analysis alone [Kanazawa et al., 1994; Le Guen et al., 1997]. This consideration is unlikely to explain the observed differences between the Japanese and European-American populations. In each study, the nucleotide sequences were determined directly from the pool of PCR-amplified products. Therefore, the derived sequence represents a consensus, with each nucleotide the one most prevalent among the pool of HCV RNAs present in the patient's serum. Thus, although this approach does not determine quasispecies distribution, differences in this distribution would be unlikely to account for the starkly divergent observations in these

geographically separate populations. It has instead been suggested that resistance to IFN is mediated by viral replication kinetics and nonspecific mutations related to the host immune response, rather than through specific sequences in the NS5a region of the genome [Pawlotsky et al., 1998]. The prototype strain does not appear to be intrinsically resistant to IFN, nor do mutant sequences appear to be intrinsically sensitive; rather, the balance between the two types of strains may be more strongly correlative with response [Pawlotsky et al., 1998]. This possibility could be tested using samples from geographically distinct areas of the world.

Our results suggest that the $NS5a_{2209–2248}$ region does not include a broadly active IFN-response element. Whether specific HCV sequences within this region may contribute to IFN sensitivity, or whether sensitivity to this agent primarily reflects the host immune response, remains unclear. Recent evidence has demonstrated that HCV resistance to IFN may be mediated through repression of the cellular protein kinase (PKR) by binding of the NS5a protein [Gale et al., 1997]; this inhibition appears be mediated by NS5a binding of the PKR dimerization domain [Gale et al., 1998]. Mutations in the $NS5a_{2209–2248}$ abrogated the ability of NS5a to bind PKR, possibly rendering HCV sensitive to the effects of IFN [Gale et al., 1998]. The demonstration that the PKR binding site on NS5a extends to HCV amino acid 2274 raises the provocative possibility that other downstream sequences may be critical in mediating NS5a inhibition. Analysis of the sequences corresponding to HCV amino acids 2249–2274 from our patient population, however, failed to show meaningful differences between responders and nonresponders (data not shown). Thus, whereas the presence of mutant NS5a sequences appears to inhibit the PKR-mediated antiviral pathway under certain conditions, their presence alone does not suffice to confer sensitivity to IFN-α. The distinct possibility that other sequences within NS5a and even outside NS5a contribute to IFN responsiveness remains. The divergence in the association between variations in NS5a sequences and IFN responsiveness in different populations suggests that responsiveness to this agent reflects a *complex* interaction between the virus and the host immune system.

Clinically useful predictors of IFN responsiveness remain elusive. Our data demonstrate, unfortunately, that analysis of viral sequences within the region encoding NS5a cannot be used to identify patients with chronic hepatitis C who are more likely to respond to treatment with IFN.

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